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Genetic drift vs. natural selection in a long-term small isolated population: major histocompatibility complex class II variation in the Gulf of California endemic porpoise (*Phocoena sinus*)

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Abstract

Although many studies confirm long-term small isolated populations (e.g. island endemics) commonly sustain low neutral genetic variation as a result of genetic drift, it is less clear how selection on adaptive or detrimental genes interplay with random forces. We investigated sequence variation at two major histocompatibility complex (Mhc) class II loci on a porpoise endemic to the upper Gulf of California, México (*Phocoena sinus*, or vaquita). Its unique declining population is estimated around 500 individuals. Single-strand conformation polymorphism analysis revealed one putative functional allele fixed at the locus *DQB* (*n* = 25). At the *DRB* locus, we found two presumed functional alleles (*n* = 29), differing by a single nonsynonymous nucleotide substitution that could increase the stability at the dimer interface of αβ-heterodimers on heterozygous individuals. Identical *trans*-specific *DQB1* and *DRB1* alleles were identified between *P. sinus* and its closest relative, the Burmeister's porpoise (*Phocoena spinipinnis*). Comparison with studies on four island endemic mammals suggests fixation of one allele, due to genetic drift, commonly occurs at the *DQA* or *DQB* loci (effectively neutral). Similarly, deleterious alleles of small effect are also effectively neutral and can become fixed; a high frequency of anatomical malformations on vaquita gave empirical support to this prediction. In contrast, retention of low but functional polymorphism at the *DRB* locus was consistent with higher selection intensity. These observations indicated natural selection could maintain (and likely also purge) some crucial alleles even in the face of strong and prolonged genetic drift and inbreeding, suggesting long-term small populations should display low inbreeding depression. Low levels of *Mhc* variation warn about a high susceptibility to novel pathogens and diseases in vaquita.

Keywords: adaptive, detrimental variation, *DQB*, *DRB*, island endemics, Mhc

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Introduction

The relative contribution of random against deterministic evolutionary forces influencing genetic variation is a crucial question in evolutionary biology (Eldredge 1989). Empirical studies confirm theoretical predictions that

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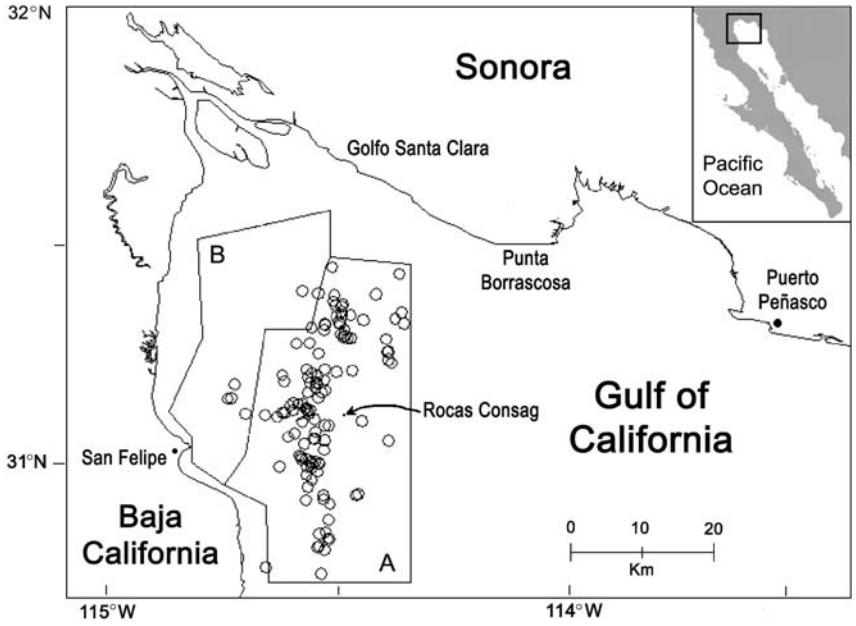

Fig. 1 Distribution of vaquita in the upper Gulf of California (Mexico). Zone 'A' is defined as 'core area' with an estimated abundance of 409 individuals (72%). Zone 'B' or 'shallow' has an estimated abundance of 158 individuals (28%). Sightings are marked as small circles. Modified from Jaramillo-Legorreta *et al*. (1999).

in long-term small isolated populations, such as island endemics, genetic drift and inbreeding have a dominant role on reducing neutral genetic variation (Frankham 1996; Frankham 1998). However, few studies address how selection on adaptive or detrimental genes interplays with random forces during hundreds or thousands of generations in these populations (Nevo *et al*. 1997; Hedrick 2001; Sommer 2005).

The vaquita or Gulf of California harbour porpoise, *Phocoena sinus*, is one of six extant species of true porpoises (Phocoenidae), and one of the smallest cetaceans (maximum length 150 cm, Vidal *et al*. 1999). The single population of this species is endemic to the upper Gulf of California, Mexico, and its abundance was estimated as 567 individuals in 1997 (Jaramillo-Legorreta *et al*. 1999). Over the second half of the past century, by-catch in gillnets reduced the population (Rojas-Bracho & Taylor 1999), and estimates suggest the decline continues with some 40 individuals removed each year (D'Agrosa *et al*. 2000). There may never have been an abundant, widespread population (Taylor & Rojas-Bracho 1999). The species was not scientifically described until 1958 (Norris & McFarland 1958), with full descriptions of external morphology not available until 1987 (Brownell *et al*. 1987). *P. sinus* has the most restricted distribution of any marine mammal, with a core area of approximately 2235 km^2 in the shallow, rich waters of the upper Gulf of California, Mexico (Jaramillo-Legorreta *et al*. 1999) (Fig. 1). They are unlike any other marine mammal in that no variability is found in the hypervariable mitochondrial control region of 43 individuals (Rosel & Rojas-Bracho 1999). Demographic simulations show that fixation of one haplotype likely resulted from historical rather than recent loss, suggesting a naturally rare species (Taylor & Rojas-Bracho 1999). Since mitochondrial genes have effective population sizes that are one quarter that of autosomal nuclear genes and consequently expected fixation times that are one quarter as large (Nichols 2001), lack of variability in mitochondrial DNA does not necessarily translate into low polymorphism and heterozygosity in the nuclear genome (Rosel & Rojas-Bracho 1999).

The major histocompatibility complex (Mhc) class II genes encode cell-surface glycoproteins that bind and present antigens from extracellular pathogens (e.g. bacteria) to T helper cells and are an essential part of the immune response of vertebrates (Klein 1986). Residues postulated to be involved in the recognition and binding of the antigens, the so-called peptide binding region (PBR), are highly variable and concentrated at the second exons of Mhc class II genes (Brown *et al*. 1993; Stern *et al*. 1994), which are among the most variable functional genes known in vertebrates (Edwards & Hedrick 1998). Studies on the evolution of Mhc genes in outbred populations typically show abundant polymorphism characterized by divergent alleles at the second exon maintained by some form of balancing selection (Bernatchez & Landry 2003; Piertney & Oliver 2006), and are considered good candidates for studies of molecular adaptation (Sommer 2005). Although a debate exists about a reduced pathogen load in marine environments (McCallum *et al*. 2004), and limited Mhc diversity on marine mammals (Trowsdale *et al*. 1989; Slade & McCallum 1992), recent sequencing analysis of populations of cetaceans reveal that the *DQB* locus of toothed whales displays substantial levels of variation consistent with positive Darwinian selection resulting in high

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levels of nonsynonymous substitutions at residues of the PBR (Murray *et al*. 1999; Hayashi *et al*. 2003; Sone *et al*. 2005; Yang *et al*. 2005; Hayashi *et al*. 2006). The only population of toothed whales surveyed at the *DRB* loci (Murray *et al*. 1999) indicates moderate levels of variation and higher nonsynonymous than synonymous substitutions at the PBR of a putative functional *DRB1* locus. On a recent study on several baleen whales (Baker *et al*. 2006), the *DQB* locus showed high nucleotide diversity (within and among species) and uninterrupted reading frames, while two *DRB* loci had low diversity among species and an apparent loss of function (Baker *et al*. 2006).

Balancing selection retards the rate of fixation of alleles and increases the level of heterozygosity in comparison with neutral predictions (Muirhead 2001; Garrigan & Hedrick 2003). Mhc alleles are typically *trans*-specific, persisting much longer than the lifetime of species (Klein *et al*. 1998; Garrigan & Hedrick 2003). However, once isolated populations become sufficiently small, natural selection become ineffective in the face of rapid genetic drift. In general, neutrality of genetic variants can be assumed when the selection coefficient *s* (either the advantage of an adaptive allele or the selective disadvantage of a detrimental one) is $< 1/2N_e$ (Kimura 1983). Weakly selected alleles are expected to be effectively neutral and can become fixed, whereas alleles with larger values of s relative to the effective population size (N_e) could be retained (+ s) or eliminated (– s) by selection (Hedrick 2001). Time to fixation for a gene under neutrality also depends on the effective population size, and according to coalescence theory, approximates 4 N_e generations for a nuclear gene (Nichols 2001). Consequently, if the vaquita population has remained small for an extended period, theory predicts drift will lead to a genome architecture characterized by low overall levels of genetic variation, except for genes under strong selection. The effects of genetic drift and natural selection on the level of adaptive genetic variation at the *DQB* and *DRB* loci were inferred for a small and highly isolated cetacean population, and compared to similar studies on island endemic mammals.

Materials and methods

Samples

Because of its inconspicuous behaviour, small individual and population size, and turbid habitat, it is very difficult to find and study vaquita in the wild (Jaramillo-Legorreta *et al*. 1999), and most of the information about its biology come from by-catch specimens recovered from fishing nets (Hohn *et al*. 1996; Vidal *et al*. 1999). We recovered skin samples from 29 by-catch individuals. For detailed information about samples, see Rosel & Rojas-Bracho (1999). Twenty-six samples were collected between 1990 and 1993, and three individuals were recovered in years 2000, 2002 and 2003, respectively. Samples included an approximately equal male/female ratio. We also included three samples from the closely related Burmeister's porpoise (*Phocoena spinipinnis*, Rosel *et al*. 1995) from central Chilean waters for comparison.

DNA isolation and polymerase chain reaction

We extracted genomic DNA by standard proteinase K digestion and organic extraction (Rosel *et al*. 1995). Two Mhc class II loci were amplified via polymerase chain reaction (PCR). A fragment of 172 bp was amplified from the second exon of the locus *DQB*, as in Murray *et al*. (1995), using primers forward 5′-CTGGTAGTTGTGTCTGCA-CAC-3′ (located at codons 14–20) and reverse 5′-CATGT-GCTACTTCACCAACGG-3′ (located at codons 78–84). Attempts to amplify the entire exon 2 of the *DRB* gene with the primers reported by Murray & White (1998) did not generate a PCR product of the correct size. These primers are forward DRBAMP-A 5′-CCCCACAGCACGTTTCTTG-3′ (Tang *et al*. 2000; located at codons 2–8) and reverse DRB3b (as appeared on Murray & White 1998) 5′-CTCGCCGCTGCATGAAAC -3′ (Ammer *et al*. 1992; located at codons 84–90). We substituted the reverse primer for GH50 5′-CTCCCCAACCCCGTAGTTGTGTCTGCA-3′ (Erlich & Bugawan 1990; located at codons 79–87) and obtained a clean, strong amplification of the expected size (210 bp, excluding primers). We used a PE 9700 thermal cycler in 50-μL volumes that contained ~80 ng genomic DNA, 1× PCR buffer, 0.2 mM each dNTP, 0.2 μM of each primer, 3 mM $MgSO_4$ and 1 U of platinum *Taq* high fidelity DNA polymerase (Invitrogen). Cycling conditions included an initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. To verify successful amplification, we visualized 5 μL of the PCR product in 1.5% agarose gels stained with SYBR Gold (Molecular Probes).

Single-strand conformation polymorphism analysis and sequencing

We screened sequence variation by single-strand conformation polymorphism (SSCP) (Orita *et al*. 1989). SSCP can detect variants separated by only a single base difference (Sunnucks *et al*. 2000). The PCR-SSCP analysis was conducted at least three times per individual sample. We mixed 3 μL of the PCR product with 15 μL of loading buffer (formamide 95%, bromophenol 0.05%, xylenecyanol 0.05%, EDTA 20 mM), denatured at 95 °C for 5 min, and then snap-cooled on ice for another 5 min before loading 8 μL on a 12% nondenaturing polyacrilamide gel (acrylamide/bis 37.5 : 1, TBE 0.5×, glycerol 10%).

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Locus	Individual	PCR	SSCP	Sequence	Alleles
DQB	UABCS-000314	15	15	7	*Phsi-DQB*01*
DRB		15	9	5	*Phsi-DRB1*0101/0102*
	UABC-V25	—	—	—	—
		15	8	5	*Phsi-DRB1*0101*
	UABC-V26	—	—	—	—
		15	8	5	*Phsi-DRB1*0101/0102*
	UABC5-7792	15	15	7	*Phsi-DQB*01*
		15	15	5	*Phsi-DRB1*0101*
	UABC6-7693	15	15	7	*Phsi-DQB*01*
		—	—	—	—
Totals	*DQB*	45	45	21	1
	DRB	60	40	20	2

Table 1 Individuals cloned for the loci *DQB* (above) and *DRB* (below), indicating number of white colonies of the correct size recovered by PCR (PCR), clones analysed by SSCP (SSCP), clones sequenced in both directions (Sequence) and identified alleles (Alleles)

Electrophoresis chamber contained TBE 0.5× previously equilibrated to 4 °C with the aid of an external cooler-recirculator (VWR 1166) set to –20 °C. We applied a constant voltage of 500 V during 15 h. After separation, gels were silver stained and captured using a digital camera. We cloned PCR products (TA cloning Kit, Invitrogen) for use as sequencing templates or as controls in the SSCP analysis. We used SSCP to screen subclones amplified using primers DRBAMP-A and GH50 with inserts of correct size. Clones with band profiles identical to that individual's genomic SSCP pattern were chosen for sequencing. The consistency of SSCP was confirmed by multiple sequencing of clones showing the identified phenotype (Table 1). We recognized heterozygotes as the sum of the SSCP profiles for two allelic subclones. We used M13 primers on an automated sequencer ABI 3730 (PE Biosystems) with BigDye Terminator to sequence selected individual subclones in both directions. We included negative controls to check for contamination in all steps.

Data analysis

Sequences were aligned by the CLUSTAL W method and translated to the deduced amino acid sequences with the software MEGALIGN (DNAstar). We used PAUP* (version 4.0b0; Swofford 1998) to construct neighbour-joining trees with the genetic distance of Jukes & Cantor (1969), and used bootstrap analysis after 1000 replications to obtain confidence estimates. We downloaded from GenBank homologous *DQB* and *DRB* sequences from other cetartiodactyls. Allele frequencies, observed and expected genotype frequencies, and deviations from Hardy–Weinberg proportions were calculated using GENEPOP 3.4 (Raymond & Rousset 1995). We also used a correction for small sample size (Nei & Roychoudhury 1974) to calculate the expected heterozygosity.

Results

Mhc variability in vaquita

We analysed by SSCP a 172-bp fragment from the second exon of the *DQB* locus in 25 individuals that represent around 5% of the estimated abundance of the species (Jaramillo-Legorreta *et al*. 1999). All individuals showed the same homozygote SSCP phenotype. Cloning and sequencing confirmed the presence of a single allele in 21 clones from three different individuals (*Phsi-DQB*01*, GenBank accession no. AY170897) (Table 1). Analysis of a 210-bp fragment in 29 individuals revealed two alleles for the second exon of the *DRB* locus (*Phsi-DRB*0101* and *Phsi-DRB*0102*, accession nos DQ914413, -14). Allele *Phsi-DRB*0101* was identified by sequence analysis of multiple clones isolated from four individuals (Table 1), and allele *Phsi-DRB*0102* was confirmed in at least two clones sequenced from two individuals (Table 1). An equal mix of the clones representing the two *DRB* alleles was loaded on the SSCP gel and reproduced exactly the phenotype of heterozygous individuals.

We observed allele *Phsi-DRB*0101* in all 29 individuals, whereas the second *DRB* allele (*Phsi-DRB*0102*) in 11 heterozygotes. No homozygotes were observed for *PhsiDRB*0102* (Table 2). The two alleles had frequencies of 0.810 and 0.189, respectively. Observed heterozygosity was higher than expected (0.379 and 0.307, respectively, Table 2), but this difference vanished when the correction for small sample size was applied (expected = 0.38). The observed genotypic numbers at the *DRB* locus were not significantly different than expected under Hardy–Weinberg proportions ($P = 0.54$, SE = 0.001), according to a probability test with the Markov chain method.

Comparison of *DQB* and *DRB* alleles with the GenBank database confirmed a high similarity with homologous cetecean *Mhc* loci. The more similar sequences for the

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Locus	N_A	Genotypes	N	Observed	Expected
DQB	1	*Phsi-DQB*01/Phsi-DQB*01*	25	1	1
DRB	2	*Phsi-DRB*0101/Phsi-DRB*0101*	18	0.620	0.656
		*Phsi-DRB*0101/Phsi-DRB*0102*	11	0.379	0.307
		*Phsi-DRB*0102/Phsi-DRB*0102*	0	0	0.035

Table 2 Number of alleles (N_A), observed genotypes, sample sizes (N), observed and expected genotypic frequencies at the Mhc class II loci from vaquita

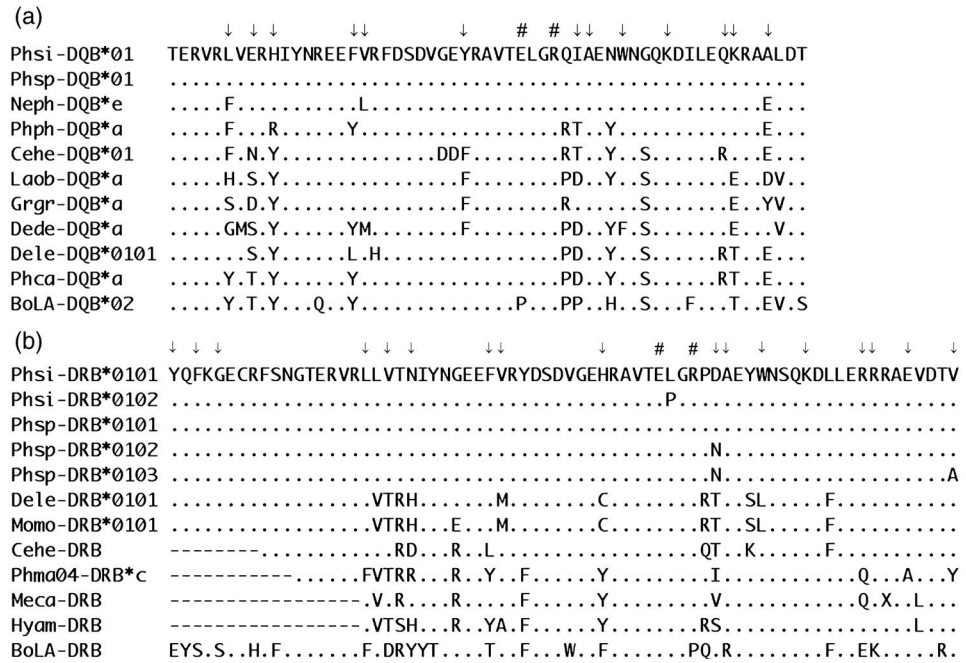

Fig. 2 Amino acid alignments of *DQB* (a) and *DRB* (b) alleles from vaquita and Burmeister's porpoises, including homologous cetacean sequences available from GenBank and *DQB* and *DRB* sequences from the cow (*BoLA*). Species codes and GenBank accession numbers are given in the legend of Fig. 3. Arrows (↓) show amino acid positions important for peptide binding (#) indicate positions in the interface of the dimer of αβ-heterodimers according to the three dimensional structure of human *HLA* DRB molecules (Brown *et al*. 1993; Stern *et al*. 1994).

vaquita *Phsi-DQB*01* allele were homologous *DQB* alleles from the finless porpoise (*Neophocaena phocaenoides*, 95–98% homology), the common porpoise (*Phocoena phocoena*, 95% homology) and the pilot whale (*Globicephala macrorhynchus*, 94% homology). The two vaquita *DRB* alleles were most similar to *DRB1* alleles from beluga (*Delphinapterus leucas*, 92–95%), narwhal (*Monodon monoceros*, 91–92% homology) and Hector's dolphin (*Cephalorhynchus hectori*, 93–94%).

Translation of alleles into amino acid sequences showed they encode uninterrupted reading frames for chains of 57 residues for the locus *DQB (*corresponding to positions 21–77, Fig. 2a) and 70 residues for the locus *DRB (*corresponding to positions 9–78, Fig. 2b). No stop codons, insertion or deletion events were observed. Between the two *DRB* alleles, a single nucleotide substitution observed at the second position of codon 53 was nonsynonymous, involving a conservative amino acid change between two nonpolar hydrophobic residues (leucine and proline, Fig. 2b). According to the three-dimensional structure of the human DR1 molecule (Brown *et al*. 1993), this position is between residues 52 and 55 postulated to form the interface of the dimer of αβ-heterodimers (also called 'superdimer'). The presence of a single nucleotide substitution prevented us from calculating synonymous and nonsynonymous ratios (d_S and d_N, respectively).

Mhc variability on Burmeister's porpoise

In the three Burmeister's porpoise samples, we found a single allele for the *DQB* locus (*Phsp-DQB*01,* accession no. DQ914412). This allele was identical to allele *Phsi-DQB*01* isolated from vaquita (Fig. 2a). At the *DRB* locus, three alleles were recovered (Accession nos DQ914415–17). No more than two *DRB* alleles were observed in the same individual, suggesting the amplification of only one locus. Allele *Phsp-DRB*0101* was identical to allele *Phsi-DRB*0101* found in vaquita. The other two alleles differed by single nonsynonymous nucleotide substitutions located in the PBR (Fig. 2b).

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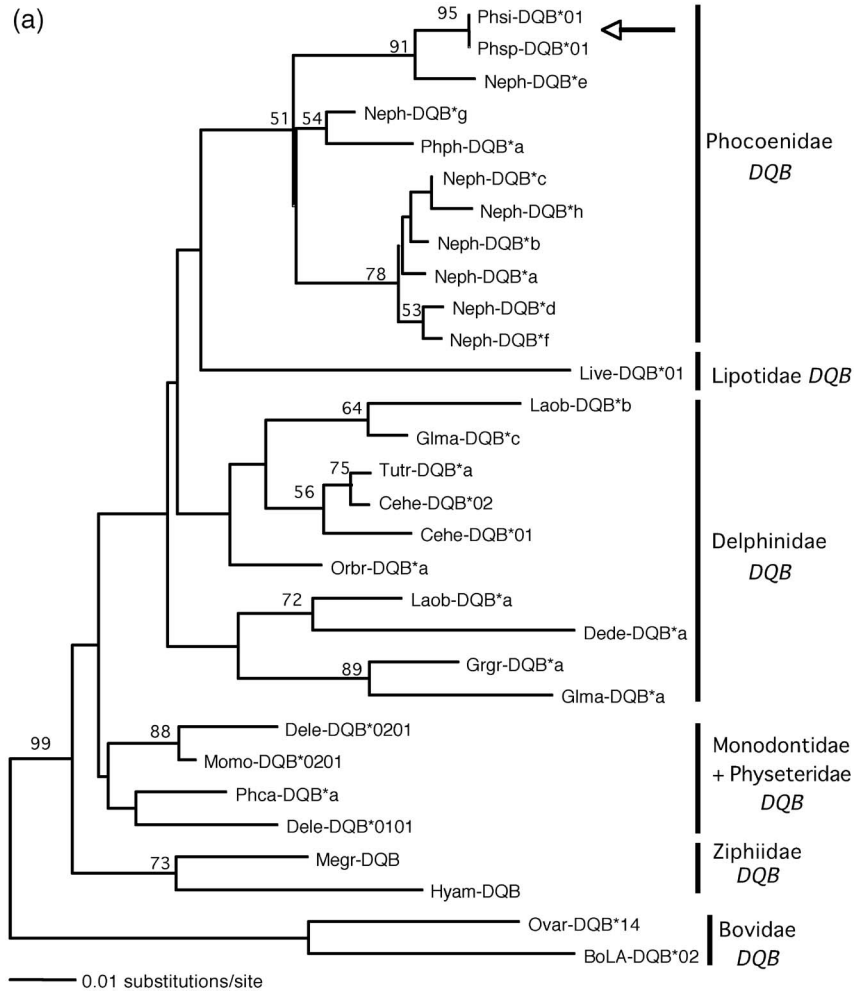

Fig. 3 Phylogenetic trees of vaquita (*Phocoena sinus*) and Burmeister's porpoise (*Phocoena spinipinnis*) *DQB* (a) and *DRB* (b) alleles. Vaquita alleles are indicated with arrows. Other homologous cetacean sequences available from GenBank were included. *DQB* sequences: harbour porpoise (*Phocoena phocoena*, AB164211), finless porpoise (*Neophocaena phocaenoides*, AB164212-19), bottlenosed dolphin (*Tursiops truncatus*, AB164221), common dolphin (*Delphinus delphis*, AB164220), Pacific white-sided dolphin (*Lagenorhynchus obliquidens*, AB164224, 25), Irrawaddy dolphin (*Orcaella brevirostris*, AB164223), short-finned pilot whale (*Globicephala macrorhynchus*, AB164226, 28), Hector's dolphin (*Cephalorhynchus hectori*, Q354628,29), beluga (*Delphinapterus leucas*, U16986, 89), narwhal (*Monodon monoceros*, U16991), sperm whale (*Physeter catodon*, AB164208), Baiji (*Lipotes vexillifer*, AY177150), northern bottlenose whale (*Hyperoodon ampullatus*, DQ354637), Gray's beaked whale (*Mesoplodon grayi*, DQ354639). Cow (*Bos taurus*, *BoLA*, U77787) and sheep (*Ovis aries*, AJ238932) sequences were used as outgroups. *DRB* sequences: Hector's dolphin (*Cephalorhynchus hectori*, DQ354675), beluga (*Delphinapterus leucas* AFO12930, -32, -34, -35, -37, -38), narwhal (*Monodon monoceros* AFO12939), sperm whale (*Physeter macrocephalus*, DQ354688), Hubb's beaked whale (*Mesoplodon carlhubbsi*, DQ354689) northern bottlenose whale (*Hyperoodon ampullatus*, DQ354679), fin whale (*Balaenoptera physalus*, DQ354674), humpback whale (*Megaptera novaeangliae*, DQ3546683), grey whale (*Eschrichtius robustus*, DQ354678), blue whale (*Balaenoptera musculus*, DQ354666), bowhead whale (*Balaena mysticetus*, DQ354670, -71), southern right whale (*Eubalaena australis*, DQ354676, -77). Cow (*Bos taurus*, *BoLA*, m30012), bison (*Bison bison* X98653) and goat (*Capra aegagrus* Z92716) sequences were used as outgroups. Bootstrap values larger than 50 are indicated above branches.

Phylogenetic analyses

Phylogenetic reconstruction of the *DQB* alleles (Fig. 3a) revealed the *trans*-species polymorphism for the identical allele found in vaquita and Burmeister's porpoise (*Phsi-DQB*01* and *Phsp-DQB*01*). This was closely related with a high bootstrap value to allele *Neph-e* from the finless porpoise. None of the deeper nodes on the *DQB* tree were supported by the bootstrap analysis.

The neighbour-joining tree of the *DRB* sequences (Fig. 3b) also revealed a *trans*-species polymorphism for the identical alleles *Phsi-DRB*0101* and *Phsp-DRB*0101*, which were more closely related to the other vaquita allele (*Phsi-DRB*0102*). The five *DRB* alleles found between the

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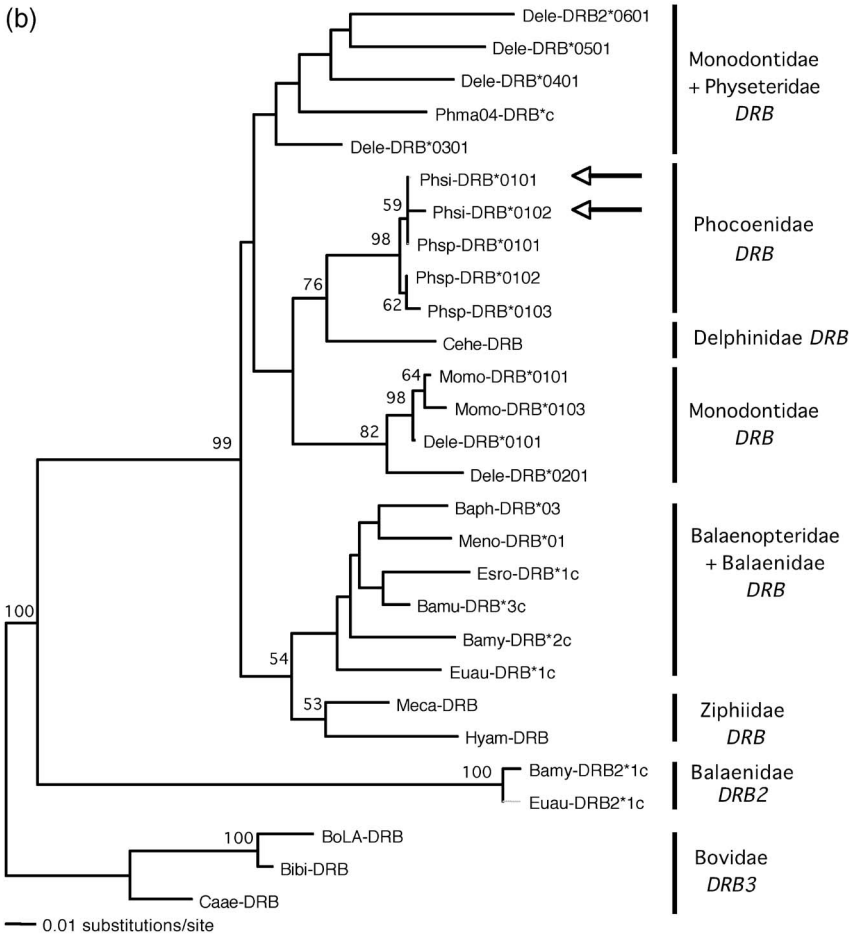

Fig. 3 *Continued*

two true porpoise species clustered together with high statistical confidence, and were significantly closely related to the *DRB* allele from Hector's dolphin (*Cehe*), which was the only available sequence from the family Delphinidae. Most of the deeper nodes of the phylogeny were not supported, with the exception of the cluster that contains putative functional *DRB* alleles that segregate with a high bootstrap value from the *DRB2* pseudogene present in Balaenidae.

Discussion

Mhc variability on vaquita

The small and isolated vaquita population had very low levels of variation at the second exon of two *Mhc* class II *B* genes. The described sequences were found in clones isolated from several different individuals (Table 1), matching the criteria for acceptance of new Mhc alleles (Kennedy *et al*. 2002). Phylogenetic analyses (Fig. 3) confirmed that the alleles from vaquita were closely related to homologous Mhc alleles from other true porpoises. Translation of *DQB* and *DRB* sequences indicated putative functional uninterrupted amino acid chains (Fig. 2). The single nucleotide substitution at position 53 found between the two vaquita *DRB* alleles was not located at the PBR, but was situated at the interface of the dimer of αβ heterodimers (Fig. 2b). Two salt bridges from glutamic acid 52 to arginine 55 on the other molecule are conserved in superdimers of human DRB1 molecules (Brown *et al*. 1993), and are conserved between the cetacean *DQB* and *DRB* amino acid sequences, including those from vaquita (Fig. 2). In some human *DP* and *DQ* variants, these residues are replaced by proline and leucine (Brown *et al*. 1993), a pattern observed at the contiguous position 53 between the two vaquita *DRB* alleles. These observations suggest an extra salt bridge could be created at position 53 of the two distinct molecules on those individuals from vaquita heterozygous for the locus *DRB*, leading to an increased stability of the superdimer on heterozygotes compared to homozygotes. Dimerization of *Mhc* class II molecules is thought to increase the specificity with T cells by simultaneously binding two T-cell receptors (Brown *et al*. 1993; Fields & Mariuzza 1996), or stabilizes weak

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T-cell receptor/CD4/class II interactions (Schafer *et al*. 1995). Several studies have demonstrated the presence of superdimers *in vivo* (Roucard *et al*. 1996; Cherry *et al*. 1998; Schafer *et al*. 1998).

On other toothed whales populations, the same *DQB* primers reveal moderate to high levels of polymorphism and putative functional alleles showing substantial nonsynonymous variation (Murray *et al*. 1999; Sone *et al*. 2005; Yang *et al*. 2005), including eight alleles in 160 finless porpoises (*Neophocaena phocaenoides*, Hayashi *et al*. 2006). Two wide surveys of sequence diversity at this locus in several other toothed and baleen whales that included single individuals for most species (Hayashi *et al*. 2003; Baker *et al*. 2006), revealed uninterrupted reading frames and extensive *trans*-specific sharing of similar alleles. To date, the few studies on the *DRB* locus of toothed whales also support the functionality suggested for the vaquita *DRB* alleles. The only previous population survey report eight alleles and extensive nonsynonymous variation for the second exon of the *DRB1* gene in 313 belugas (*Delphinapterus leucas*), and three alleles in 11 narwhals (*Monodon monoceros*) (Murray *et al*. 1999). *DRB1* sequences from single individuals of four toothed whales indicated continuous reading frames and a lack of indels, suggesting this gene is an orthologue of the ancestral functional ruminant gene *DRB3* (Baker *et al*. 2006). This contrast with the *DRB1* and *DRB2* loci of baleen whales that displayed low diversity among species and indels that caused loss of function or interruption of reading frames on most sequences (Baker *et al*. 2006).

The effect of recent bottlenecks on Mhc variation

The low levels of Mhc class II variation observed in vaquita are uncommon even for other mammalian species that have experienced recent population bottlenecks. For example, the critically endangered baiji (*Lipotes vexillifer*), reduced to less than 100 individuals in the past decades shows 43 alleles at several duplicated *DQB* loci (Yang *et al*. 2005), and a survey on the endangered giant panda (*Ailuropoda melanoleuca*) recently reduced to 1000 individuals revealed seven *DRB* alleles that averaged 20% amino acid divergence (Wan *et al*. 2006). These argue against the role of the recent population decline on vaquita (related to by-catch on gillnets) on the reduced levels of variation observed at the Mhc class II loci. According to a generation time of 10 years in vaquita (Taylor & Rojas-Bracho 1999), the population decline should have started only five or six generations ago. Assuming that the most recent population estimate exceeding 500 individuals (Jaramillo-Legorreta *et al*. 1999) represents the lowest population to date, and considering approximately half of those individuals are breeding adults with an equal sex ratio (Hohn *et al*. 1996), we obtain a rough estimate, likely an upper limit, of actual $N_e = 250$. However, considering N_e can reach 1/10 of the census size on wild populations (Frankham 1995), a lower limit for N_e would be 50 individuals. Based on these estimates of effective size, fixation of a neutral nuclear gene in the population is expected after 4 N_e generations (Nichols 2001), which represents at least some 200–1000 generations. This likely represents an underestimate, because balancing selection over Mhc loci could retard the rate of fixation one or two orders of magnitude (Klein *et al*. 1998; Muirhead 2001). Thus, to explain the fixation of the *DQB* locus in vaquita, a scenario of long-term small population size during at least some 2000–10 000 years needs to be invoked.

Mhc variation on long-term small populations

If the paucity of Mhc class II gene diversity found in vaquita is related to long-term evolutionary process, island endemic mammalian species with similar evolutionary and demographic histories (i.e. *in situ* evolution through isolation during thousand years sustaining small population sizes), should show a similar pattern at homologous Mhc class-II loci. Four species were chosen for comparison based on availability of data, including two rodents (Australian bush rat, *Rattus fuscipes greyii*; Malagasy giant jumping rat, *Hypogeomys antimena*), the California Channel island fox, *Urocyon littoralis*, and the Hawaiian monk seal, *Monachus schauinslandi* (Table 3). With the exception of the bush rat, the other three species (and the vaquita) declined recently related to human activities and are considered threatened at different levels (Table 3, IUCN 2004). The island fox, the monk seal and the vaquita sustain current effective sizes of hundreds of individuals. The giant jumping rat has effective sizes of few thousand individuals, and a similar scenario seems likely for the bush rat. As populations remain small and isolated, the ability for selection to retain adaptive genetic variation is expected to decrease, particularly at those loci subjected to low selection intensities (Hedrick 2001). Based on the above estimates of effective size for vaquita, the selection coefficient estimated for the fixed *DQB* locus is < 0.002 ($N_e = 250$) or < 0.01 ($N_e = 50$). Considering the selection coefficient estimated for the human *DQB* locus (0.0085, Satta *et al*. 1994), the intensity of genetic drift at the lower estimate of effective size is enough that the locus is expected to behave as neutral and eventually become fixed as observed. Among the other island endemics (Table 3) fixation of a single allele at the *DQB* is observed on four islands for the island fox, including the three with the smallest effective sizes. At the *DQA* locus, thought to be subject to even less selection intensity ($s = 0.0028$, Satta *et al*. 1994), the Hawaiian monk seal and nine populations from the Australian bush rat are also fixed for a single allele. The Malagasy giant jumping rat had only two alleles

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Table 3 Genetic variation at Mhc class II loci and neutral markers for island endemic mammals. For each species, common and scientific name and conservation status (IUCN 2004), island name, island area or species range, effective size (N_e) and island age (million years ago, Ma) are included. The information on all studies was derived from sequence analysis. For Mhc loci, all data refer to the second exon, except for locus *DQA* in *H. antimena* which includes the second intron and third exon, For Mhc loci included are locus name, length in base pairs (bp) of the DNA fragment analysed, total sample sizes (*N*), number of alleles observed in each island, the observed heterozygosity in parentheses, and (if applicable) total number of alleles (*A*), segregating sites (*S*), average percentage nucleotide (%*N*) and amino acid (%*A*) divergence between alleles. In parentheses are the minimum and maximum number of nucleotide (or amino acid, respectively) differences observed between pairs of alleles, and total different predicted amino acid chains or phenotypes (P). For the mitochondrial control region (d-loop). length of the fragment (bp) analysed, sample size (*N*), number of haplotypes on each island, and in parenthesis the gene diversity (*), nucleotide diversity (^), or percentage of variable sites (#), and total number of haplotypes (*H*) are included. For microsatellite loci, the number of loci analysed, sample size (N) and for each island the number of alleles and in parenthesis the observed heterozygosity are included. NA, data not available. References: (1) Sommer 2003; (2) Seddon & Baverstock 1999; (3) Hinten *et al*. 2003; (4) Armstrong 1995; (5) Kretzmann *et al*. 2001; (6) Kretzman *et al*. 1997; (7) GenBank accessions AF093799, AY007203, AY007204; (8) Aguilar *et al*. 2004; (9) Gilbert *et al*. 1990; (10) Rosel & Rojas-Bracho 1999; (11) Rosa *et al*. 2005.

Species and status	Island	Area or range (km²)	N_e	Island age (Ma)	*DQ*	*DRB*	D-loop	Microsatellites	Reference
Malagasy giant jumping rat	Madagascar	< 200	1840	NA	*DQA*				
Hypogeomys antimena	Northern				162 bp, *N* = 247	217 bp, *N* = 229	465 bp, *N* = 97	NA	(1)
Endangered	Southern		6900		2 (0.39)	4 (0.38)	2 (0.07)*		
					2 (0.47)	5 (0.55)	4 (0.29)*		
					A = 2	*A* = 5	*H* = 4		
					S = 4	*S* = 37			
					%*N* = 2.4 (4)	%*N* = 9.03 (3–31)			
					%*A* = 0 (0)	%*A* = 18.7 (6–21)			
					P = 1	P = 5			
Australian bush rat			NA		*DQA*	NA			
Rattus fuscipes greyii					249 bp, *N* = 305		365 bp, *N* = 305	6 neutral loci, *N* = 242–305	(2,3)
Low risk	Dog	0.6		0.0077	1 (0)		1 (0)	15 (0.19)	
	Eyre	11		0.006	1 (0)		3 (0.002)^	21 (0.42)	
	Goat	3.03		0.006	1 (0)		1 (0)	17 (0.35)	
	Gre enly	2.02		0.0105	1 (0)		1 (0)	12 (0.51)	
	Hopkins	1.62		0.0084	1 (0)		1 (0)	17 (0.38)	
	Kangaroo	4000+		0.0095	9 (0.64)		10 (0.017)^	53 (0.83)	
	Lacy	3.6		0.0084	1 (0)		1 (0)	7 (0.03)	
	Masillon	1.8		0.0077	1 (0)		1 (0)	11 (0.24)	
	Neptune	2.43		0.0119	1 (0)		1 (0)	12 (0.4)	
	N Gambier	0.64		0.0091	1 (0)		2 (0.001)^	15 (0.37)	
	Pearson	2.13		0.0105	2 (0.04)		1 (0)	19 (0.44)	
	Waldegrave	2.92		0.006	2 (0.52)		2 (0.004)^	19 (0.52)	
	Williams	1.41		0.0091	2 (0.56)		1 (0)	27 (0.51)	
					A = 21		*H* = 27		
					S = 48				
					%*N* = 6.6 (9–28)				
					%*A* = 12.3 (1–19)				
					P = 21				

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Table 3 *Continued*

Species and status	Island	Area or range (km²)	N_e	Island age (Ma)	*DQ*	*DRB*	D-loop	Microsatellites	Reference
Hawaiian monk seal *Monachus schauinslandi* Endangered	Kure Atoll Pearl and Hermes Lisianski Laysan F. Frigate	NA	140†	3.5–4‡	*DQA* 169 bp, *N* = 25 1 (0) *DQB* 141 bp, *N* = 27 2 (NA) *S* = 18 %*N* = 12.8 (18) %*A* = 21.3 (10) P = 2	NA	359 bp, *N* = 50 1 2 2 3 2 *H* = 3 (0.6%)#	3 neutral loci, *N* = 46–108 2 (0.2) 2 (0.62) 2 (0.48) 2 (0.34) 2 (0.38)	(4,5,6,7)
California channel island fox *Urocyon littoralis* Critically Endangered	S Miguel S Rosa S Cruz S Nicolas S Catalina S Clemente	37 217 249 58 194 145	163 955 984 247 979 551	0.016 0.016 0.016 0.0022 0.0022 0.0008–0.00043	*DQB* 174 bp, *N* = 152 1 (0) 1 (0) 2 (0.21) 1 (0) 4 (0.55) 1 (0) *A* = 4 *S* = 7 %*N* = 1.7 (1–4) %*A* = 3.5 (2) P = 3	267 bp, *N* = 152 1 (0) 2 (0.16) 2 (0.14) 2 (0.36) 3 (0.36) 1 (0) *A* = 3 *S* = 29 %*N* = 7.5 (13–24) %*A* = 12.7 (9–14) P = 3	NA	18 neutral loci, 3 flanking MHC, *N* = 152 1.78 (0.11), 4 (0.42) 2.56 (0.21), 6.3 (0.51) 2.39 (0.22), 4.6 (0.58) 1 (0), 2.6 (0.50) 2.61 (0.36), 5.3 (0.41) 2.11 (0.26), 4 (0.59)	(8,9)
Gulf of California porpoise (vaquita) *Phocoena sinus* Critically Endangered	Upper Gulf of California	2235	50–250	2–3‡	*DQB* 171 bp, *N* = 25 1 (0)	210 bp, *N* = 29 2 (0.37) *S* = 1 %*N* = 0.5 (1) %*A* = 1.5 (1) P = 2	322 bp, *N* = 43 1 (0)	2 neutral loci, *N* = 7 'Multiple alleles at each locus, most individuals heterozygous' 11 Neutral loci *N* = 1 'Only 2 loci showed heterozygosity'	(10, 11) This tudy

†Calculated considering N_e = 1/10 of the census size. ‡Estimated age of the species.

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at the *DQA* differing by three intron and one silent exon nucleotide substitution. Overall, fixation at a *DQ* loci was observed at 15 of the 22 (68.1%) island populations compared (the giant jumping rat and the Hawaiian monk seal were each considered as single populations, Table 3). These observations suggest a dominant role of random genetic drift over natural selection at the *DQ* alleles of island endemic mammals. The remaining seven populations compared sustained two and up to four alleles. Two large populations from the island fox retained two and four alleles for *DQB*, respectively, but the alleles are highly similar at both the nucleotide and amino acid level (Table 3). Divergent *DQB* alleles typically observed in most outbreed mammals (Bontrop *et al*. 1999; Yeager & Hughes 1999), including cetaceans (Hayashi *et al*. 2003; Baker *et al*. 2006), were present only at the *DQB* locus of monk seal, and among the *DQA* locus in four populations of the more abundant bush rat (Table 3).

In contrast, it is estimated that the selection intensity at the human *DRB* locus ($s = 0.019$, Satta *et al*. 1994) is at least two times higher than for *DQB*. Consequently, theory predicts selection could maintain functional variation more effectively at the *DRB* than at the *DQB*, even in the face of strong and prolonged genetic drift and inbreeding. In the vaquita population, balancing selection could have maintained the two different *DRB* alleles differing by a single nonsynonymous substitution based on the proposed selective scenario of an increased stability of the superdimer on heterozygous individuals. In this case, the selection coefficient of the low frequency allele was estimated as > 0.002 ($N_e = 250$) or > 0.01 ($N_e = 50$). However, two alternate scenarios regarding the rare *DRB* allele should be considered: (i) a relatively recent neutral mutation drifting in the population, and (ii) an effectively neutral allele that emerged in an ancestral species and has been retained in vaquita. The observed Hardy–Weinberg equilibrium supports the first scenario. However, detection of ongoing selection is not easy even with large sample sizes (Garrigan & Hedrick 2003), and we expect a reduced statistical power on our ability to detect deviations from equilibrium, based on the small sample size and low polymorphism observed. On other hand, the *trans*-specific polymorphism between vaquita and Burmeister's porpoise suggest at least one *DQB* and one *DRB* allele have been maintained, presumably by balancing selection, over the last 2 or 3 millions years since the estimated split of the two lineages (Rosel *et al*. 1995). This period of time is at least two orders of magnitude larger than the expected coalescence time of a neutral nuclear allele on the vaquita population, suggesting the second scenario is unlikely unless we consider a much more recent divergence between the two species (e.g. during the last glacial periods), which contradicts previous molecular estimates (Rosel *et al*. 1995) and seems improbable considering the geological history of the actual Gulf of California over the last 3 million years (Ledesma-Vázquez 1999).

On the other island endemics mammals (Table 3), balancing selection at the *DRB* locus retained functional polymorphism among six of the eight populations compared from three different species (vaquita, giant jumping rat and island fox), and fixation was observed on only two populations (25%). The vaquita and three populations of the island fox had only two alleles, and another population from the island fox showed three alleles (Table 3). The characteristics of the *DRB* polymorphism retained in these populations generally agreed with the expected intensity of genetic drift based on the effective sizes of the populations. Contrasting with the two *DRB* alleles from vaquita differing by a single residue, island fox alleles show moderate amino acid divergence on comparatively larger populations. The relatively abundant giant jumping rat retained five highly divergent *DRB* alleles (Table 3). Only two populations from the island fox were fixed for a single allele, including the smallest island with the lowest effective size.

The maintenance of polymorphism within populations is dependent on the product of selection intensity, mutation rate and effective population size (Nevo *et al*. 1997; Hedrick 2001). The strength of selection acting at the *DQ* loci was generally insufficient to maintain variation in small populations isolated during hundred or thousand generations (Table 3). However, on most cases some functional variation was still retained at the *DRB* locus, consistent with higher selection intensity at this locus. This agrees with theoretical models (Nevo *et al*. 1997) that suggest moderate or strong balancing selection on small populations can oppose random drift and maintain polymorphism during thousands of generations. On other nonisland (outbreed) mammals, higher numbers of alleles are usually reported at the locus *DRB1* compared to *DQA1* and *DQB1* (e.g. Bontrop *et al*. 1999; Yeager & Hughes 1999), an observation that supports the presumed differences on selection intensities between these loci. The relationship between Mhc heterozygosity and neutral loci is complex and commonly inconsistent across different species (Bernatchez & Landry 2003). However, a reduced *Mhc* polymorphism has been correlated with low genome-wide genetic variation (Hedrick 2001; Sommer 2005). In the island-endemics compared, most of bush rat populations and the vaquita showed one fixed mitochondrial haplotype, and the rest had few closely related haplotypes. Also, limited polymorphism was observed at nuclear neutral hypervariable markers (Table 3). High mutation rates at microsatellite loci (Table 3) likely prevented fixation (except for the San Nicolas's island fox), but most populations from the monk seal and the island fox had only two alleles and reduced heterozygosities. The bush rat showed much more alleles

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consistent with larger effective sizes. Preliminary analyses of microsatellite loci in vaquita indicate the presence of distinct alleles and some level of heterozygosity (Table 3). The microsatellite loci flanking Mhc genes on the island fox displayed higher polymorphism compared to the other markers, a pattern consistent with the joint influence of selection at linked loci and mutation rate.

Detrimental variation on long-term small populations

In historical small populations, some slightly deleterious alleles are also effectively neutral and theory predicts they can occur at high frequencies or get fixed (Hedrick 2001). An unusually high frequency of limb and axial malformations on vaquita empirically support this prediction. A sixth extra digit (polydactyly) is present on the third metacarp of both pectoral fins in each of 43 individuals analysed (Ortega-Ortiz *et al*. 2000). Other anomalies include hyperosteosis in 23% ($N = 62$), and of these about half (55%) also present fusion of vertebrae 26 and 27 (Ortega-Ortiz *et al*. 2000). These anatomical malformations could be linked in a syndrome (Ortega-Ortiz *et al*. 2000) and result from the pleiotropic effects of mutations on upstream genes (Galis *et al*. 2001). In the vaquita population, these harmful alleles of little effect likely correspond to recessive alleles (dominance $h = 0.2$) with selection coefficients $s < 0.01$ ($N_e = 250$) or < 0.05 ($N_e = 50$). Until now, there is no evidence that this syndrome causes any serious impairment to the survival or reproduction of individuals, which agrees with the hypothesis that its effects on fitness should be small (possibly hidden from selection) and become fixed during the evolutionary history of the species. Although they contribute to the genetic load, they likely do not significantly increase the inbreeding depression (Hedrick 2001).

In contrast, when small populations have become inbred over an extended period, detrimental alleles causing moderate to high effects on fitness are likely purged, since the increase in homozygosity resulting from inbreeding exposes recessive deleterious alleles to selection, reducing the inbreeding depression (Hedrick 2001). In general, the conditions pertaining to naturally rare populations translate into gradual inbreeding over long periods, which have been proposed as the conditions for efficient purging (Taylor & Rojas-Bracho 1999; Keller & Waller 2002).

Trans-*species polymorphism*

It as been suggested that *Phocoena sinus* is a relict population of an ancestral species related to the Burmeister' porpoise (*Phocoena spinipinnis*) (Norris & McFarland 1958; Barnes 1985; Rosel *et al*. 1995). The Burmeister's porpoise is currently distributed along the Pacific and Atlantic coasts of South America, with its closest populations located on central Peruvian waters. The presence between these two species of an identical *trans*-specific *DQB* allele and at least one *DRB* allele strongly confirm their common ancestry. Generally, *trans*-species evolution is taken as evidence for the long-term retention of allelic lineages by balancing selection (Klein *et al*. 1998). An ancestral population of *P. spinipinnis* is thought to have crossed the equator during one of the Pliocene or Pleistocene cooling periods and became trapped in the more temperate waters of the northern Gulf of California as water temperatures later rose (Norris & McFarland 1958; Rosel *et al*. 1995). The isolated population then likely evolved *in situ*, either through fragmentation of a larger ancestral population, or a founder event (Taylor & Rojas 1999). Founder events at species inception, natural periods of population contraction and expansion (caused for instance by disease outbreaks or by natural changes on available island habitat related to sea level changes during glaciations), along with long-term small population size, are three not mutually exclusive demographic scenarios that could synergistically contribute to a significant erosion of genetic diversity in island endemics. Given that they show very low overall levels of genetic variation (Table 3), the particular contribution of different past demographic events would be difficult to estimate accurately with current available markers.

Conservation implications

Low levels of genetic variation at Mhc genes warn about a low adaptive potential and high susceptibility of the whole population to novel infectious diseases (O'Brien & Evermann 1988; Hughes 1991). Additionally, long-term small populations may take longer to reach the carrying capacity, and be slower to recover from catastrophes (Frankham *et al*. 2002). Studies by Aguilar *et al*. (2004) and Weber *et al*. (2004b) support the notion that few Mhc alleles differing by single nonsynonymous nucleotide substitutions may be crucial for the survival of isolated populations or species. Although *Mhc* alleles do show a degree of specificity, a single *Mhc* molecule can bind multiple peptides that have common amino acids at particular anchor positions (Altuvia & Margalit 2004). Besides, the polymorphism observed at the *DRB* locus in vaquita suggested superdimers could be important on triggering the immune response. To date, there has not been any report of infectious disease in vaquita, and its parasite load is not unusually high or uncommon (Vidal *et al*. 1999).

Low genome-wide genetic variation, or even lack of, is a common characteristic of many long-term small isolated populations, and is the result of standard evolutionary processes (i.e. genetic drift). However, this does not necessarily translate into a species or population being doomed to extinction. Many highly inbred populations have fully recovered after population declines (Groombridge *et al*.

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2000; Jamieson *et al*. 2006), even in the presence of low levels of Mhc variation (Mikko *et al*. 1999; Weber *et al*. 2004b; Babik *et al*. 2005). In particular with marine mammals, some studies indicate they are able to overcome histories of long-term population bottlenecks and maintain their persistence in the ecosystem (Weber *et al*. 2004a). Their viability seems to depend mainly on factors outside their genetic makeup, such as human-induced mortality, in the specific case of vaquita by-catch in gillnets.

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